

# The integrin $\alpha\beta6$ drives pancreatic cancer through diverse mechanisms and represents an effective target for therapy

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## Abstract

Pancreatic ductal adenocarcinoma (PDAC) has a 5-year survival rate of less than 4% and desperately needs novel effective therapeutics. Integrin  $\alpha\beta6$  has been linked with poor prognosis in cancer but its potential as a target in PDAC remains unclear. We report that transcriptional expression analysis revealed that high levels of  $\beta6$  mRNA correlated strongly with significantly poorer survival ( $n = 491$  cases,  $p = 3.17 \times 10^{-8}$ ). In two separate cohorts, we showed that over 80% of PDACs expressed  $\alpha\beta6$  protein and that paired metastases retained  $\alpha\beta6$  expression. *In vitro*, integrin  $\alpha\beta6$  promoted PDAC cell growth, survival, migration, and invasion. Treatment of both  $\alpha\beta6$ -positive human PDAC xenografts and transgenic mice bearing  $\alpha\beta6$ -positive PDAC with the  $\alpha\beta6$  blocking antibody 264RAD, combined with gemcitabine, significantly reduced tumour growth ( $p < 0.0001$ ) and increased survival (log-rank test,  $p < 0.05$ ). Antibody therapy was associated with suppression of tumour cell activity (suppression of pErk growth signals, increased apoptosis seen as activated caspase-3) and suppression of the pro-tumorigenic microenvironment (suppression of TGF $\beta$  signalling, fewer  $\alpha$ SMA-positive myofibroblasts, decreased blood vessel density). These data show that  $\alpha\beta6$  promotes PDAC growth through both tumour cell and tumour microenvironment mechanisms and represents a valuable target for PDAC therapy.

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## Introduction

Pancreatic ductal adenocarcinoma (PDAC) is the fifth leading cause of cancer-related death with a 5-year survival rate of less than 4% [1] largely due to the asymptomatic nature and late-stage presentation of the disease. While new chemotherapy regimens, such as FOLFIRINOX (fluorouracil, leucovorin, irinotecan,

and oxaliplatin) and gemcitabine with nab-paclitaxel (nanoparticle albumin-bound paclitaxel), have provided significant but incremental increases in overall survival (increasing to 11.1 and 8.5 months, respectively) compared with standard gemcitabine chemotherapy (6.7 months for gemcitabine alone [2,3]), the need to identify more effective therapeutic interventions remains paramount.

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In a small ( $n=34$ ) early study [4] it was noted that the integrin  $\alpha\text{v}\beta 6$  was expressed by PDAC. Integrins regulate cellular proliferation, adhesion, migration, and invasion, and their deregulated expression and signalling can promote cancer progression [5]. Integrins constitute a superfamily of 24 heterodimeric cell-surface receptors, composed of  $\alpha$  and  $\beta$  subunits, which modulate cell behaviour through bi-directional signalling between the intracellular and extracellular compartments [6]. In most normal tissues,  $\alpha\text{v}\beta 6$  expression is weak or absent, but is up-regulated *de novo* by cells undergoing tissue remodelling, including in carcinogenesis [7]. Moreover, the epithelial-specific integrin  $\alpha\text{v}\beta 6$  previously has been described as a poor prognostic marker in multiple cancers [8–11]. However, a report by Hezel *et al* [12] showed that antibody inhibition of  $\alpha\text{v}\beta 6$  in a transgenic PDAC mouse model (Ptf1-Cre;LSL-Ras<sup>G12D/+</sup>;p53<sup>fl/+</sup>) seemed to promote PDAC progression, raising doubts as to its potential as a therapeutic target. As studies in many other cancers [8–11] suggested that  $\alpha\text{v}\beta 6$  promoted cancer, and as novel pancreatic cancer therapies are needed desperately, we have examined the role of  $\alpha\text{v}\beta 6$  clinically, functionally, and therapeutically in PDAC and conclude that it should be pursued as a promising target for PDAC therapy.

## Materials and methods

### Clinical samples and mRNA expression data

The human tissues used in this study were obtained under ethical permissions local to the source of materials. All clinical data were analysed according to REMARK guidelines [13]. A total of 265 PDAC clinical samples, as two separate tissue microarray (TMA) slide sets, were provided by The University of Verona, Italy, and The Beatson Institute, Scotland, UK. Matched primary and metastatic PDAC samples were supplied by the Rapid Autopsy Programme, The University of Nebraska. Eight individual cohorts of mRNA expression data, totalling 491 patients, were collected from the Barts Cancer Institute Pancreatic Expression Database, ICGC PDAC cohort (Biankin), University of Glasgow (Glasgow), TCGA PDAC cohort (PAAD), as well as previously published datasets [14–17].

### Cell lines and antibodies

KRas-mutant CFPac1, Panc04.03, and Panc1 human pancreatic cancer cell lines and the human pancreatic stellate cell line (PS1 [18]) were grown as adherent monolayers. Cells were grown in a 100% humidified atmosphere of 8% (volume/volume) of carbon dioxide ( $\text{CO}_2$ )/air. Cell lines were tested routinely for, and found free of, *Mycoplasma*. All cancer cell lines were grown in Roswell Park Memorial Institute (RPMI)-1640 medium or Dulbecco's modified Eagle's media (DMEM) (PAA Laboratories, Fisher Scientific UK, Loughborough, UK)

supplemented with 10% foetal calf serum (FCS) (Biosera, Labtech International, East Sussex, UK) and glutamine (Sigma-Aldrich, Dorset, UK; 4 mM final concentration). Human pancreatic stellate (PS1) cells were grown in a 1:1 mixture of DMEM (E15-843; PAA Laboratories) and Ham's F12 (E15-817; PAA Laboratories) supplemented with 10% FCS, glutamine (4 mM final concentration), and 1  $\mu\text{g}/\text{ml}$  puromycin (P9620; Sigma), as a selection agent. The genetic identity of all human lines was confirmed by STR profiling (LGC Standards, Teddington, Middlesex, UK).

All clinical samples were labelled with mAb 6.2G2 (anti- $\beta 6$ ; a gift from Biogen Idec, Boston, MA, USA) as previously described [8]. The  $\alpha\text{v}\beta 6$ -blocking mouse antibody 10D5 (MAB2077Z; Sigma-Aldrich) was purchased and the rat mAb 53A.2 was created by JFM at Barts Cancer Institute, London. Antibody 264RAD ( $\alpha\text{v}\beta 6$ -blocking with some  $\alpha\text{v}\beta 8$ -blocking activity [19]) and IgG control for pre-clinical studies were supplied by Oncology iMED, AstraZeneca, Cambridge, UK.

### Flow cytometry and FACS

Flow cytometry was performed as previously described [8]. Briefly,  $2 \times 10^5$  cells were incubated with 10  $\mu\text{g}/\text{ml}$  of primary antibody in suspension and incubated on ice for 45 min. Controls included unstained cells and class-matched IgG primary antibodies. The appropriate secondary AlexaFLUOR (Molecular Probes, Thermo Fisher, Loughborough, UK) antibodies were added at 1:250 and incubated for 30 min at 4°C. For flow cytometry, 10 000 events were acquired by flow cytometry on a FACS Calibur cytometer with Cell Quest Pro software version 4.0.2 (BD Biosciences, San José, CA, USA).

### *In vitro* cell proliferation assays

The optimised number of cells (3000–5000 depending on cell line) were seeded per well, in quadruplicate wells, of 96-well plates and the following day,  $\alpha\text{v}\beta 6$  function blocking antibodies or control antibody were added at 0, 0.2, 1, 5, and 10  $\mu\text{g}/\text{ml}$  final concentration. After 4 days at 37°C, fresh antibody was added to the cells at the same concentration and left until day 7. On day 7, the medium was replaced with 100  $\mu\text{l}$  of MTT reagent (M5655; Sigma-Aldrich) and generation of formazan product was quantified according to the manufacturer's instructions. Experiments were conducted with four replicates and repeated at least three times.

### Transwell® migration and invasion assays

For Transwell® assays,  $5 \times 10^4$  cells were seeded per well post-treatment into 6.5 mm diameter, 8  $\mu\text{m}$  pore-size Transwells® (Corning BV, Thermo Fisher). For migration assays, the underside of the wells was coated with fibronectin (Sigma-Aldrich) at 10  $\mu\text{g}/\text{ml}$  or TGF $\beta$ 1 LAP (Sigma-Aldrich) at 1  $\mu\text{g}/\text{ml}$  final

concentration. For invasion assays, the upper surface of the wells was coated with 70 µl of BD Matrigel Basement Membrane matrix (Matrigel™; BD Biosciences):media (1:2 ratio). After 16 h (migration) or 72 h (invasion), cells that had migrated/invaded were trypsin/EDTA harvested and counted using an automated cell counter CASY (Scharfe Systems, Midland, Ontario, Canada) as published previously [20].

### Immunohistochemistry

Harvested tumours from mice were formalin-fixed and processed to paraffin wax. Sections were cut at 5 µm thickness, dewaxed, and endogenous peroxidases were blocked with a 0.45% solution of H<sub>2</sub>O<sub>2</sub> in methanol for 15 min. Antibodies to Ki67 (ab92742; Abcam, Cambridge, UK; 1:200 final dilution), cleaved caspase-3 (9664S; Cell Signaling Technology, London, UK; 1:100 final dilution), phospho-ERK (4376S; Cell Signaling Technology; 1:100 final dilution), endomucin (sc-65495; Santa Cruz Biotechnology, Dallas, TX, USA; 1:200 final dilution), cytokeratin (ZO622; Dako, Agilent Technologies, Stockport, UK; 1:500 final dilution), α-SMA (MO851; Dako; 1:300 final dilution), phospho-Smad3 (ab52903; Abcam; 1:100 final dilution), and Smad4 (sc-7966; Santa Cruz Biotechnology; 1:300 final dilution) were used to immunostain tumours using a standard avidin–biotin complex technique (Vectastain Elite ABC Kit; Vector Laboratories, Peterborough, UK). Picro-Sirius red (CI 35782, Sigma-Aldrich) was used to assess collagen deposition in tumours. Slides were scanned (Pannoramic Digital Slide Scanner, 3DHISTECH Ltd, Budapest, Hungary) and tumour staining was analysed on the Pannoramic Viewer software (version 1.15.2; 3DHISTECH Ltd) using the NuclearQuant module for Ki67, Smad4, and pErk, and the DensitoQuant module for cleaved caspase-3, cytokeratin, and phospho-Smad3. The number of endomucin-positive blood vessels was counted using the Ariol 'Angiosight' Image Analysis module (Ariol SL-8; Leica Microsystems, Wetzlar, Germany), and NIH ImageJ freeware (<https://imagej.nih.gov/ij/download.html>) was used to quantify collagen deposition and α-SMA expression. Three independent observers performed blind scoring of the multiple cores for each cancer using the following scoring system: αvβ6 intensity staining was scored out of 4 (0: absent; 1: background staining; 2: weak; 3: moderate; 4: strong); and the percentage of epithelial cells staining positively was scored out of 4 (1: < 25%; 2: 25–50%; 3: 51–75%; 4: 76–100%), where the combined score gave a score in the range of 0–8. Median scores were determined and used for statistical analyses. We noted that the Beatson TMA stained much more weakly than the Verona TMA, possibly suggesting some degradation of the target antigen. Patients with a score greater than or equal to the median score of 3 were considered positive for αvβ6; 5 or greater was considered strong and the remaining patients with a median score of less than 3 were considered negative.

### Pre-clinical animal studies

All animal experiments followed UK Home Office Guidelines determined by the Animals (Scientific Procedures) Act 1986. For human xenograft model development, 8-week-old female CD1 nu/nu mice (Charles River Laboratories, Harlow, Essex, UK) were inoculated subcutaneously (200 µl in the flank) or orthotopically (40 µl into the tail of the pancreas) with either  $1 \times 10^6$  CFPac1 alone or  $1 \times 10^6$  CFPac1 in combination with  $2 \times 10^6$  PS1 cells in PBS. Orthotopic xenograft tumours were harvested 6 weeks post-injection, while subcutaneous tumours were harvested 4 weeks post-injection. For the xenograft antibody therapy study, 8-week-old female CD1 nu/nu mice were obtained from Charles River Laboratories, and  $1 \times 10^6$  CFPac1 in combination with  $2 \times 10^6$  PS1 cells were injected subcutaneously. Tumours were measured with callipers bi-weekly in two directions, where volume was calculated using the formula  $(\text{width}^2 \times \text{length})/2$ . When tumours reached 100 mm<sup>3</sup>, mice were randomised to receive 4 weeks of bi-weekly intraperitoneal injections (200 µl) of either 10 mg/kg human IgG or 264RAD, gemcitabine (Gemzar) at 100 mg/kg, or a combination of 100 mg/kg gemcitabine and 10 mg/kg 264RAD ( $n=9$  per treatment). Transgenic KDC (PdxCre+KRas<sup>LSL-G12D/+</sup>dup6<sup>-/-</sup>; described in the supplementary material, Figure S3) mouse studies were performed at the CRUK Beatson Institute, Glasgow, UK. To recapitulate the clinical setting, KDC animals showing signs of sickness (tented stance, palpable tumours) received bi-weekly intraperitoneal injections (200 µl) of either 10 mg/kg human IgG plus 100 mg/kg gemcitabine, or 10 mg/kg 264RAD plus 100 mg/kg gemcitabine. Mice were culled when signs of severe sickness were evident. Mice were sacrificed before tumours reached Home Office volume limits, at signs of sickness, or after 6 weeks of therapy.

### Statistical analysis

The mRNA abundance data were pre-processed as outlined previously [21]. In brief, each cohort was normalised and pre-processed independently. Pair-wise differences between antibody-treated versus control *in vitro* assays and immunohistochemical analysis of tumours were tested using Student's *t*-test. Treatment differences between *in vivo* tumour growth treatments were tested using a Wald test from a normal linear mixed model fitted by maximum likelihood [22]. A proportional hazards model was used to compare *in vivo* survival, with a log-rank (score) tested for an overall difference and a Wald test between groups. Analysis was performed in the statistical software Prism GraphPad (Version 5.0b; GraphPad Software, San Diego, CA, USA) and R (R Development Core Team, 2010 2.15.1) with the nmle package. A *P* value equal to or less than 5% was considered statistically significant.



## Results

High  $\beta 6$  mRNA levels correlate with poor overall survival in PDAC

Eight independent cohorts of PDAC gene expression datasets, totalling 491 PDAC cases, were each dichotomised into high (top 25%; red line, Figure 1) and low (bottom 75%; black line, Figure 1) expressers based on *ITGB6* mRNA expression levels (mRNA abundance for each cohort is recorded in the supplementary material, Table S1). Combined data show that with high expressers of *ITGB6* (red line;  $n = 125$ ), there was a significantly lower overall survival compared with weak expressers of *ITGB6* (black line;  $n = 366$ ) [hazard ratio (HR): 2.07; 95% confidence interval (CI): 1.59–2.69; log-rank test:  $p = 3.17 \times 10^{-8}$ ] (Figure 1, lower histogram).

$\alpha\text{v}\beta 6$  protein is expressed by most PDACs and is retained by metastases

Two separate cohorts of PDAC samples were analysed independently for expression of  $\alpha\text{v}\beta 6$ . The Beatson cohort had 118 PDAC cases and 12 normal pancreata, whereas the Verona dataset had 147 patients and 20 normal pancreata. Examples of typical staining are shown in Figure 2A. Expression of  $\alpha\text{v}\beta 6$  was restricted specifically to epithelial cells as observed previously [7,8]. Normal pancreas samples were mostly negative for  $\alpha\text{v}\beta 6$ , though occasionally some duct cells expressed  $\alpha\text{v}\beta 6$  weakly. Expression of  $\alpha\text{v}\beta 6$  was detected in 98% (median score  $\geq 3$ ) of Verona samples and 83% of Beatson samples, although staining intensity was uniformly weaker on the Beatson TMA and thus staining may be an underestimate. Using a median score of  $\geq 5$  to represent strong staining (as shown on the PanIN and PDAC in Figure 2A), 84% of Verona PDAC samples expressed  $\alpha\text{v}\beta 6$  strongly. We then examined six PDAC patients for whom we had primary tumour tissue and their matched metastatic tumours from lung, colon or liver; all the primary tumours expressed  $\alpha\text{v}\beta 6$  strongly and all metastases were also  $\alpha\text{v}\beta 6$ -positive, 70% retaining similarly strong expression to that of the matched primary (Figure 2B and Table 1).

Blockade of  $\alpha\text{v}\beta 6$  reduces proliferation, migration, and invasion of  $\alpha\text{v}\beta 6$ -positive pancreatic cancer cells *in vitro*

Integrin  $\alpha\text{v}\beta 6$  was expressed on seven out of nine pancreatic tumour cell lines, as determined by flow cytometry (see supplementary material, Table S2). The  $\alpha\text{v}\beta 6$ -blocking antibodies 53A.2 (Figure 3A) and 264RAD (Figure 3B) similarly and significantly reduced the proliferation of  $\alpha\text{v}\beta 6$ -positive, but not  $\alpha\text{v}\beta 6$ -negative, PDAC cancer cells. Both antibodies also reduced the migration of  $\alpha\text{v}\beta 6$ -positive Panc04.03 and CFPac1 pancreatic cancer cells towards their ligand LAP (Figure 2C,D). 10D5

( $\alpha\text{v}\beta 6$ -blocking) and 264RAD also reduced the invasion of these  $\alpha\text{v}\beta 6$ -positive cells through Matrigel<sup>TM</sup>-coated Transwells<sup>®</sup> (Figure 3E,F), compared with control-treated cells and  $\alpha\text{v}\beta 6$ -negative Panc1 cells. Thus,  $\alpha\text{v}\beta 6$  promotes PDAC cancer cell growth, migration, and invasion.

264RAD antibody therapy reduces the growth of pancreatic human xenograft tumours

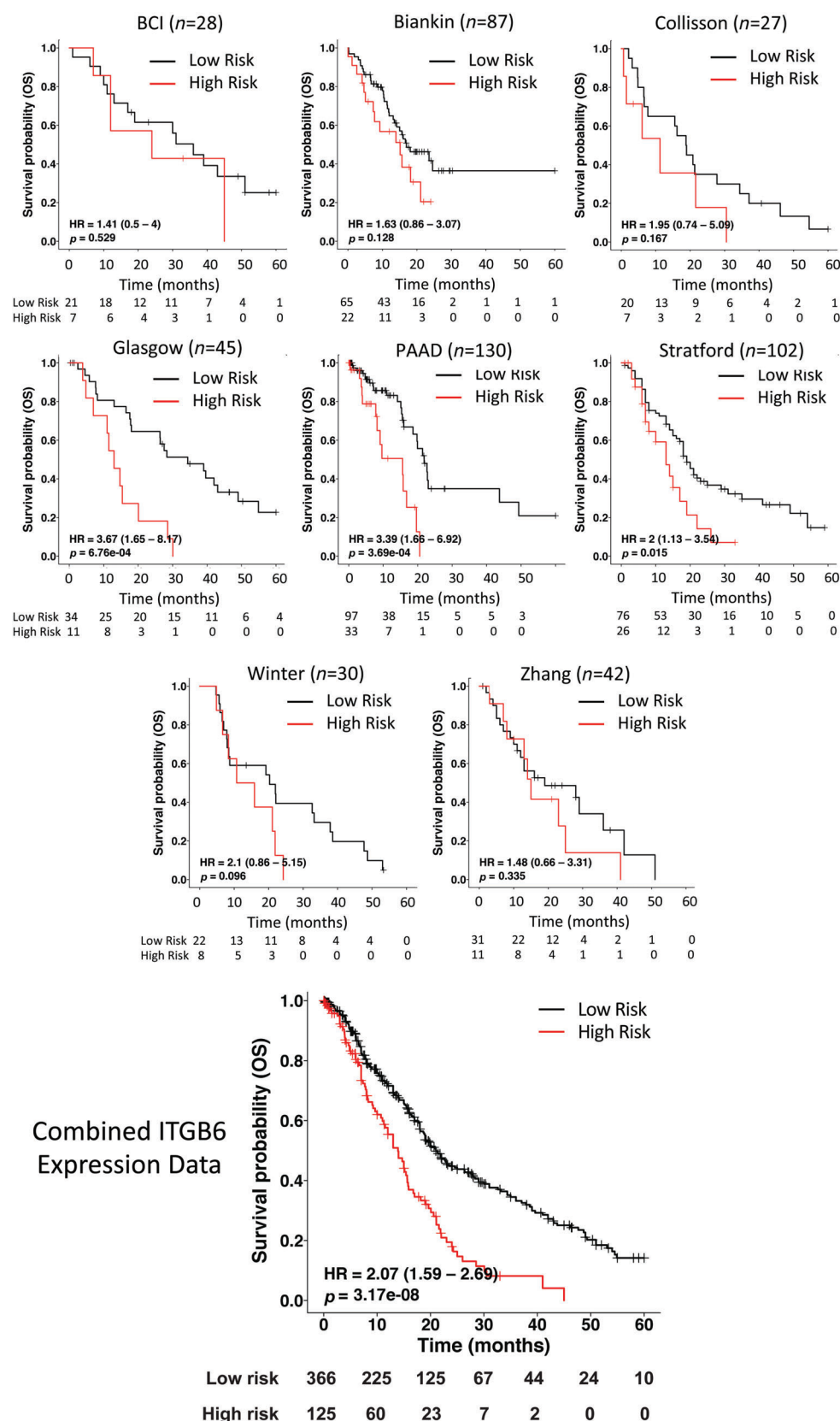
CFPac1 cells were injected alone or in combination with a human pancreatic stellate cell (PSC) line (PS1) orthotopically or subcutaneously. The presence of PSCs reduced the number and density of endomucin-positive blood vessels and increased collagen deposition compared with CFPac1 cells growing alone (see supplementary material, Figure S1). Since both the subcutaneous and the orthotopic tumours with PSCs developed similar hypovascular and desmoplastic stroma seen in human pancreatic cancer [23,24], we used the more easily tractable subcutaneous xenograft model for the  $\alpha\text{v}\beta 6$ -blocking 264RAD [19] antibody therapy studies *in vivo*.

Treatment of mice bearing 100 mm<sup>3</sup> CFPac1/PS1 subcutaneous tumours with 264RAD demonstrated significantly reduced tumour growth, compared with isotype control ( $p \leq 0.0001$ ) (Figure 4A). Treatment of tumours with gemcitabine alone also significantly reduced tumour growth compared with isotype control ( $p \leq 0.0001$ ), or when compared with 264RAD monotherapy ( $p = 0.012$ ). However, the combination of 264RAD with gemcitabine demonstrated the greatest reduction in tumour volume of all mice during treatment, compared with isotype control ( $p \leq 0.0001$ ), or compared with gemcitabine alone ( $p = 0.028$ ) (Figure 4A and see supplementary material, Figure S2). In fact, the tumours in three mice treated with 264RAD plus gemcitabine vanished completely (see supplementary material, Figure S2). No toxicity (loss of weight, change in appearance or behaviour) was observed in any mice during any treatment (data not shown).

To determine the molecular mechanisms associated with 264RAD-induced tumour reduction, immunohistochemistry demonstrated a significant down-regulation of proliferation (Ki67) and growth signalling (phosphorylated Erk), and a significant up-regulation of apoptosis (cleaved caspase-3; Figure 4B) in the 264RAD-treated tumours compared with control-treated tumours ( $p \leq 0.0001$ ) (Figure 4C).

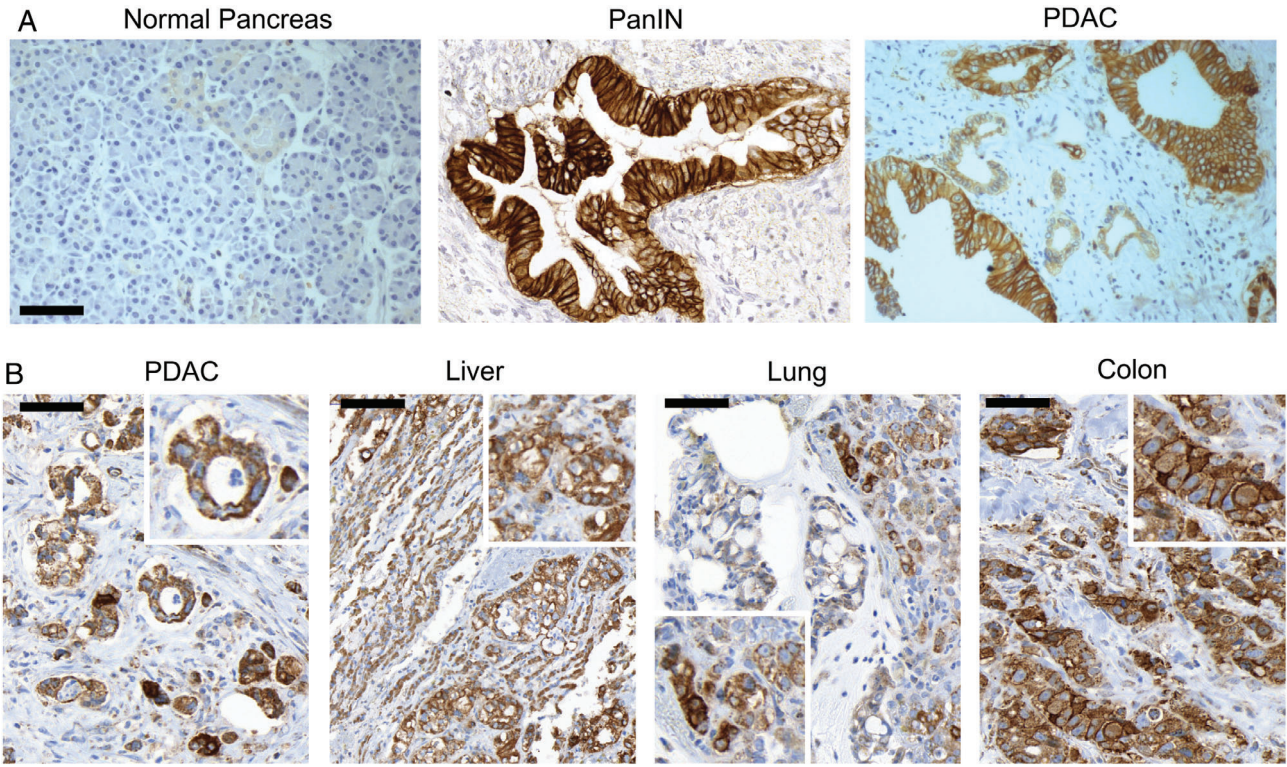
Therapy with the  $\alpha\text{v}\beta 6$ -blocking 264RAD antibody significantly improves survival in immunocompetent transgenic mice bearing  $\alpha\text{v}\beta 6$ -expressing PDAC tumours

We sought to determine the effect of 264RAD antibody therapy on PDAC using immunocompetent transgenic mice. The pathological development of PDAC in well-established KPC (PdxCre<sup>+</sup>KRas<sup>LSL-G12D/+</sup>p53<sup>LSL-R172H/+</sup>) mice closely



**Figure 1.** Expression of  $\alpha v\beta 6$  at the mRNA level in human PDAC. Kaplan–Meier survival curves of PDAC patients based on integrin  $\beta 6$  gene (*ITGB6*) expression at the mRNA level from eight cohorts: Barts Cancer Institute (BCI) pancreatic expression database, ICGC PDAC cohort (Biankin), University of Glasgow (Glasgow), TCGA PDAC cohort (PAAD), as well as previously published datasets (Collisson, Stratford, Winter, and Zhang [14–17]). The red line indicates the upper quartile of *ITGB6*-positive patients and the black line indicates *ITGB6*-weakly positive or -negative patients. The lower histogram shows the combined Kaplan–Meier survival analysis of the total of 491 PDAC patients based on integrin  $\beta 6$  mRNA (*ITGB6*) expression. Again, the red line indicates the upper quartile of *ITGB6*-positive patients ( $n = 125$ ) and the black line *ITGB6*-weakly positive or -negative patients ( $n = 366$ ). Hazard ratio (HR) = 2.07 (95% CI 1.59–2.69); log-rank test  $p = 3.17 \times 10^{-8}$ .





**Figure 2.** Expression of  $\alpha\beta6$  protein in human PDAC. FFPE PDAC tissues were labelled with mAb 6.2G2 to assess  $\alpha\beta6$  expression. (A) Representative images of human normal pancreas, PanIN, and invasive PDAC tumour tissue stained for  $\alpha\beta6$  expression are shown. Scale bar = 100  $\mu$ m. (B) Representative staining for  $\alpha\beta6$  expression in a primary tumour and paired metastases from a single patient is shown, with an enlarged inset for improved clarity. Scale bar = 50  $\mu$ m.

**Table 1.** Expression of  $\alpha\beta6$  in primary PDAC and matched metastases

	Tumour site	Cases	Strong $\alpha\beta6$ expression	Moderate, weak, nil $\alpha\beta6$ expression
Primary	Pancreas	6	6 (100%)	0 (0%)
Metastases	Liver	6	3 (50%)	3 (50%)
	Lung	2	2 (100%)	0 (0%)
	Colon	5	4 (80%)	1 (20%)

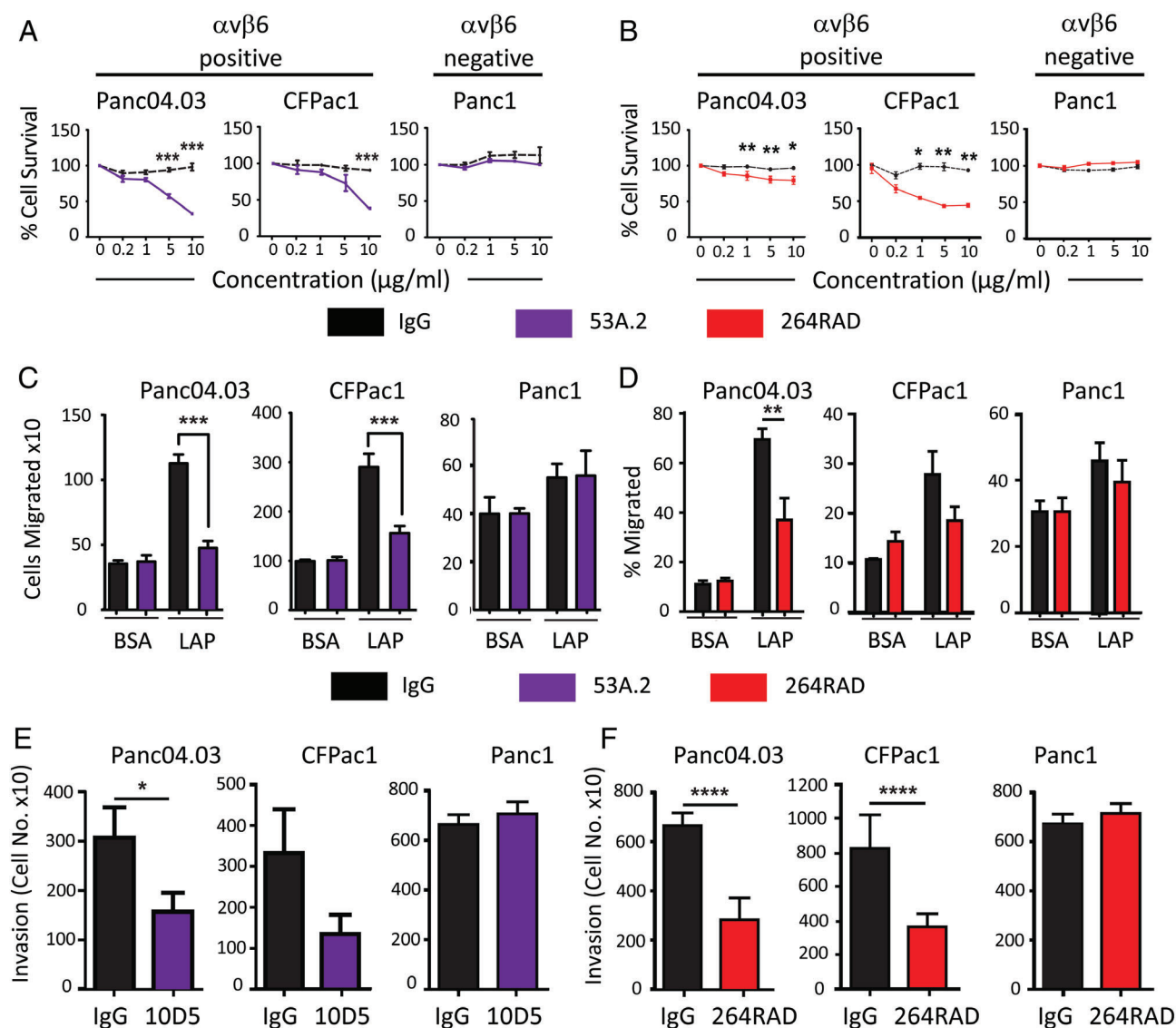
Sections of primary PDAC tumours and patient-matched secondary tumours were collected from six patients (generous gift from the Rapid Autopsy Programme, The University of Nebraska) and stained for expression of  $\alpha\beta6$  using immunohistochemistry. All primary tumours expressed  $\alpha\beta6$  strongly (median score  $\geq 5$ ) and all metastases retained  $\alpha\beta6$  expression, 70% retaining strong expression.

matches the progression of human disease [25]. However, unlike in humans where  $\alpha\beta6$  is expressed on most human PDACs (see above), integrin  $\alpha\beta6$  was detected only in PanIN lesions but not in the PDAC primary tumours of KPC mice (data not shown). Therefore, we examined a new transgenic pancreatic cancer model called KDC (PdxCre<sup>+</sup>KRas<sup>LSL-G12D/+</sup>dusp6<sup>-/-</sup>), whose mice possess the same activating mutation in KRas as KPC mice together with deletion of the dual-specificity phosphatase 6 (dusp6) targeted to the pancreas. These mice develop PanIN lesions which progress to invasive, metastatic PDAC between 3 and 10 months of age (see supplementary material, Figure S3). Similar to human disease, we discovered that KDC mice demonstrated a striking expression of  $\alpha\beta6$  in PanINs and primary tumours (Figure 5A). Mirroring human therapy, in

which therapeutic intervention is usually administered in late-stage disease, KDC mice with palpable pancreatic tumours and displaying visible symptoms of sickness were treated with gemcitabine in combination with 264RAD or isotype control antibody. The 264RAD-treated mice showed significantly increased overall survival in comparison with isotype control treated animals (log-rank test  $p = 0.028$ ; HR: 4.92; 95% CI: 1.04–23.28) (Figure 5B). Immunohistochemistry revealed that 264RAD treatment of PDAC-bearing KDC mice significantly reduced tumour cell proliferation (Ki67), tumour growth signalling (pErk), blood vessel density (endomucin), and TGF $\beta$  signalling (nuclear Smad4), and showed a trend towards reduced phosphorylated Smad3,  $\alpha$ SMA, and collagen deposition (Figure 5C,D).

## Discussion

Cytotoxic pharmaceuticals remain the principal tool for therapy of PDAC and while there have been promising developments that offer incremental increases in survival of additional months [2,3] patients still need vastly improved therapies if we are to significantly improve upon the 5-year survival rate of less than 4%. Our data suggest that combining blockade of integrin  $\alpha\beta6$  with cytotoxic therapy could significantly help to achieve that goal.

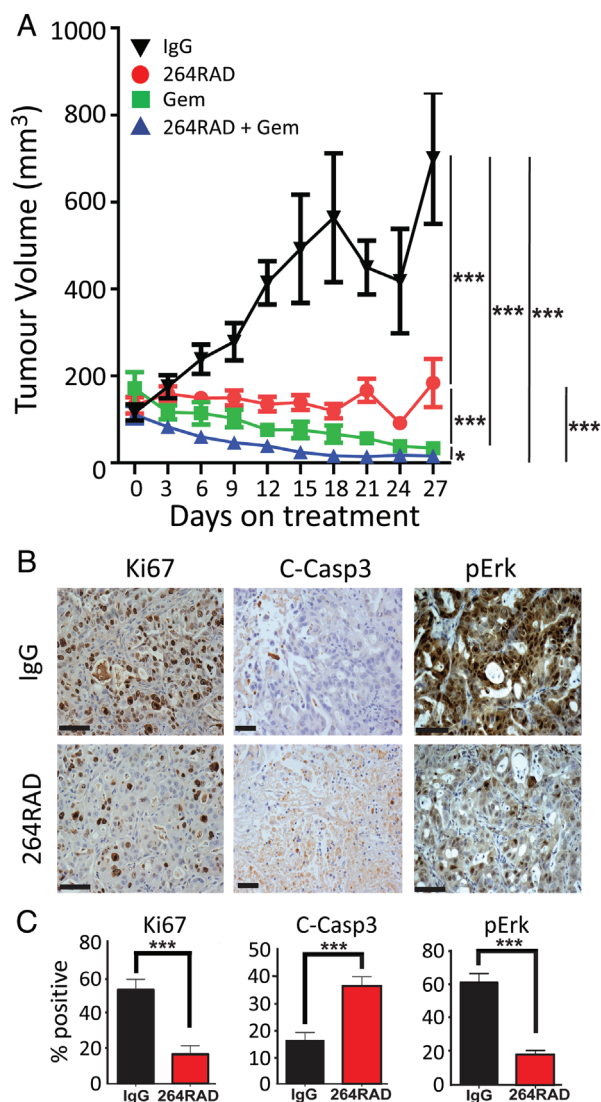


**Figure 3.** Antibody blockade of  $\alpha v \beta 6$  inhibits proliferation, migration, and invasion of PDAC cell lines. Proliferation of Panc04.03, CFPac1, and Panc1 cells treated with varying concentrations of  $\alpha v \beta 6$  function-blocking antibodies, (A) 53A.2 (purple) or (B) 264RAD (red), versus IgG control (black). Two-way ANOVA (Bonferroni's correction), \* $p < 0.05$ ; \*\* $p < 0.01$ ; \*\*\* $p < 0.001$ . Migration of PDAC cells towards the latency-associated peptide (LAP) of TGF $\beta$ , compared with BSA control, following incubation with (C) 53A.2 (purple) or (D) 264RAD (red) versus IgG control (black) used at the same concentration. Data show the mean  $\pm$  SEM of three independent experiments performed in quadruplicates. Two-way ANOVA, \*\* $p < 0.005$ ; \*\*\* $p < 0.001$ . Invasion of PDAC cells through Matrigel™ following treatment with (E) 10D5 (purple) or (F) 264RAD (red) compared with IgG control antibody (black) used at the same concentration. The number of invaded cells was counted after 72 h. Data show the mean  $\pm$  SEM of three independent experiments performed in triplicate. Student's  $t$ -test, \* $p < 0.05$ ; \*\*\*\* $p < 0.0001$ .

We, and others, have reported that high expression of the pro-invasive integrin  $\alpha v \beta 6$  correlates with poor overall survival from cancer [8–11]. Thus, when Sipos *et al* reported [4] that all of their 34 PDAC cases analysed expressed  $\alpha v \beta 6$ , it was reasonable to assume that this integrin would also promote this disease. However, a robust study from Hezel *et al* [12] suggested that  $\alpha v \beta 6$  was a tumour suppressor in PDAC, probably through local activation of TGF $\beta$ . It is well established that TGF $\beta$  can be both tumour-suppressive and tumour-promoting (reviewed in refs 26 and 27). A major mechanism by which TGF $\beta$  suppresses normal epithelial growth is through activation of cyclin-dependent kinases p21 and p15. However, carcinoma cells often

develop mutations that make them refractory to this growth suppression and allowing them to respond directly to the ability of TGF $\beta$  to promote invasion or indirectly by TGF $\beta$ -dependent generation of a tumour-permissive microenvironment [26]. Hezel *et al* used the Ptf1-Cre;LSL-Ras<sup>G12D/+</sup>;p53<sup>fl/+</sup> mice that develop PDAC relatively early, at 9–10 weeks [12]. They reported that antibody blockade of  $\alpha v \beta 6$  (or TGF $\beta$ ) promoted early development of PanINs and progression to PDAC, but had no effect on the tumour microenvironment, and that these observations correlated with TGF $\beta$  signalling and  $\alpha v \beta 6$  expression. Thus, their data are consistent with the conclusion that the transformed pancreatic ductal cells in Ptf1-Cre;LSL-Ras<sup>G12D/+</sup>;p53<sup>fl/+</sup>





**Figure 4.** 264RAD therapy in the CFPac1/PS1 subcutaneous human xenograft mouse model of pancreatic cancer. (A) A total of  $0.5 \times 10^6$  CFPac1 cells and  $1 \times 10^6$  PS1 cells were co-injected subcutaneously into female CD1nu/nu mice. When tumours reached approximately 100 mm<sup>3</sup>, mice were entered into four different treatment arms: IgG isotype control antibody (10 mg/kg); 264RAD (10 mg/kg); gemcitabine (100 mg/kg); or 264RAD (10 mg/kg) and gemcitabine (100 mg/kg) ( $n=9$  per group). Animals were treated bi-weekly for 4 weeks. Data were analysed using a linear mixed model fitted to log volume [22]; \* $p < 0.05$ ; \*\*\* $p < 0.0001$ . (B) Representative images and (C) quantification of IgG-treated and 264RAD-treated tumours stained for Ki67, cleaved caspase-3, and phosphorylated ERK (pERK). Student's  $t$ -test, \*\*\* $p < 0.0001$ . Scale bar = 50  $\mu$ m.

mice retain the TGF $\beta$ -dependent growth-suppressive signalling pathways even when they have developed into PanINs and PDAC. Another mechanism that may be relevant was reported by David *et al* [28] who showed that PDAC cells that retained functional SMAD4 signalling underwent TGF $\beta$ -dependent EMT that transitioned to apoptosis driven by the repurposing of the transcription factor Sox4. Thus, in this situation, inhibition of  $\alpha\beta6$ -dependent activation of TGF $\beta$  would reduce apoptosis and enhance PDAC survival. However, the number of PDAC tumours retaining TGF $\beta$ -dependent

SMAD signalling is likely to be a smaller fraction of humans with PDAC, since most human PDACs have genetic dysfunction in TGF $\beta$  signalling [26,27].

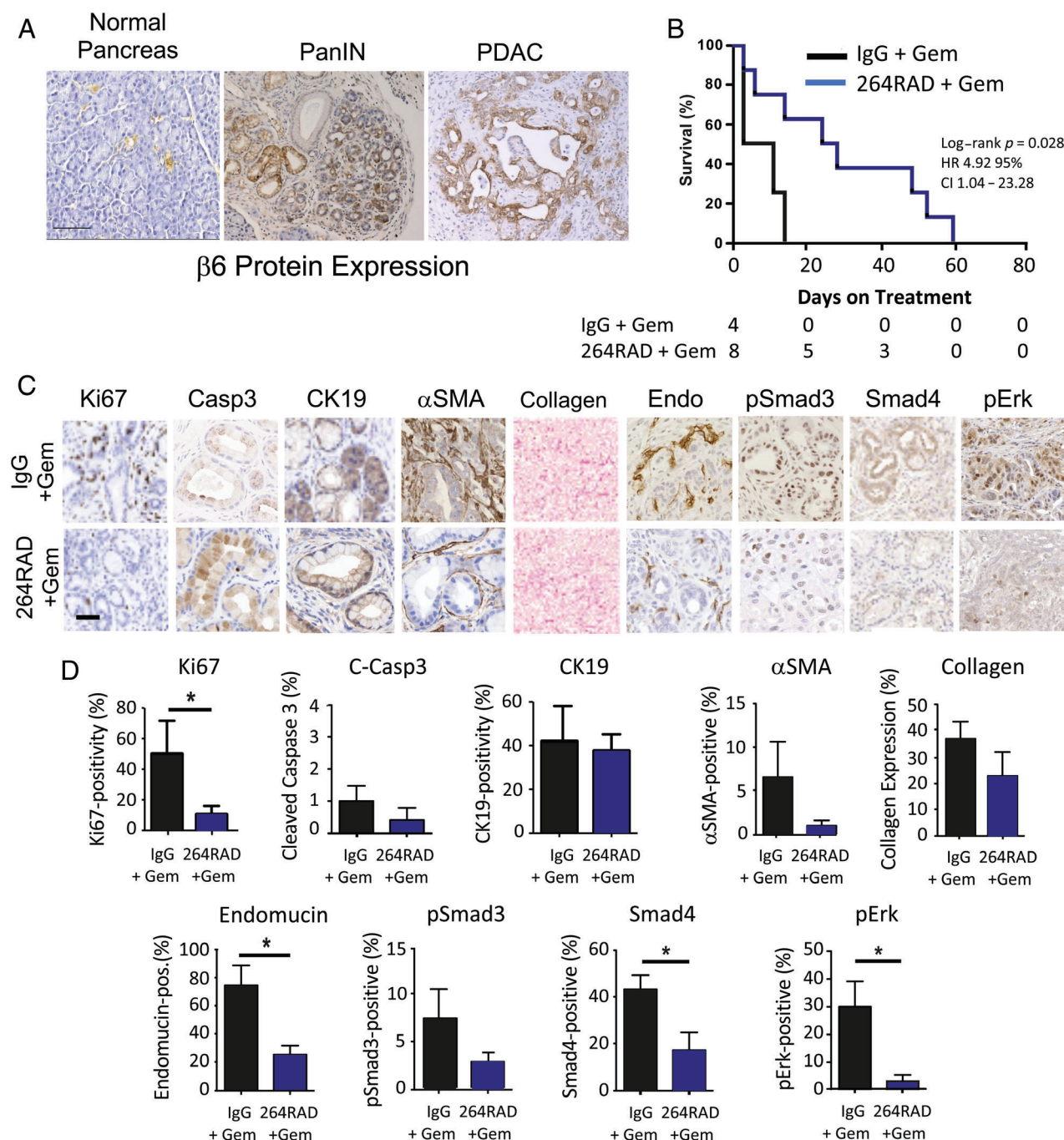
Analysis of the expression of *ITGB6* mRNA on eight separate cohorts showed that high (top 25% quartile) expression, in general, correlated with poorer survival. When the data from all 491 PDAC cases were analysed, the high expression group was associated with significantly poorer survival (Figure 1; HR: 2.07; log-rank test:  $p = 3.17 \times 10^{-8}$ ). Thus, there is a clear association linking  $\beta6$  and survival from PDAC. Our data also showed that in addition to most PDACs expressing  $\alpha\beta6$  on their surface, the integrin is retained by metastases, so therapies directed to this integrin would target both primary and secondary disease.

To investigate the role of  $\alpha\beta6$  in PDAC experimentally, we examined a panel of PDAC cell lines and confirmed that seven of nine expressed  $\alpha\beta6$  (see supplementary material, Table S2). When we grew  $\alpha\beta6$ -expressing (Panc0403 and CFPac1) and  $\alpha\beta6$ -negative (Panc1) cell lines in the presence of two different  $\alpha\beta6$ -blocking antibodies (53a2 and 264RAD), we noted that, compared with control IgG, both  $\alpha\beta6$ -blocking antibodies suppressed growth (Figure 3A,B). This is consistent with the report of Singh *et al* [29] that the *ITGB6* gene is required for *Ras*-oncogene-dependent growth of PDAC cells. We further showed that  $\alpha\beta6$  promoted migration and invasion (Figure 3C–F), confirming that, as has been shown for other cell types [8,20,30],  $\alpha\beta6$  imparts greater proliferative, migratory, and invasive capacity to PDAC cells.

To establish whether  $\alpha\beta6$  could be an effective therapeutic target, we tested two novel PDAC tumour models that reflect human disease. We generated a xenograft from combined injection of human PDAC and pancreatic stellate cells (CFPac1/PS1) that developed desmoplastic, hypoangiogenic tumours, mimicking human disease [23]. Treatment with 264RAD, which blocks  $\alpha\beta6$  function, stopped established tumours growing and enhanced gemcitabine therapy ( $p < 0.0001$ ), even curing 33% of mice in the combination therapy group (see supplementary material, Figure S3). Mechanistically, the 264RAD antibody increased apoptosis and blocked pErk growth signals and tumour cell division (Figure 4C). Thus, just as we have reported in breast and oral tumours [8,19] inhibition of  $\alpha\beta6$  with 264RAD could abrogate and sometimes even eliminate established PDAC tumours.

The human xenograft studies required immunodeficient mice and thus we turned to a new transgenic PDAC mouse model, KDC (PdxCre<sup>+</sup>KRas<sup>LSL-G12D/+</sup>dusp6<sup>-/-</sup>), which combines a *Ras*<sup>G12D</sup> mutation with loss of dual-specific phosphatase 6 (Dusp6), considered a potential tumour suppressor in PDAC [31]; in KDC mice,  $\alpha\beta6$  was expressed in PanINs and PDAC tumours (Figure 5). Blockade of  $\alpha\beta6$  with 264RAD in combination with gemcitabine significantly (HR: 4.92; log-rank test  $p = 0.028$ ) increased survival of KDC mice (Figure 5). This is in agreement with our human xenograft study but is in contrast to the report by Hezel





**Figure 5.** Treatment of PDAC-bearing KDC mice with 264RAD and immunohistochemical analysis. (A) Representative immunohistochemical analysis of  $\alpha v \beta 6$  expression in normal, PanIN, and PDAC from PdxCre<sup>+</sup>KRas<sup>LSL-G12D/+</sup>Dusp6<sup>-/-</sup> (KDC) mice. Normal pancreas shows no  $\alpha v \beta 6$  on acinar cells and weak staining on some ductal cells. Strong expression of  $\alpha v \beta 6$  protein was evident in PanIN and PDAC. (B) Kaplan–Meier survival curve of KDC mice treated with IgG isotype control (10 mg/kg) and gemcitabine (100 mg/kg) (grey line;  $n = 4$ ), or 264RAD (10 mg/kg) and gemcitabine (100 mg/kg) (purple line;  $n = 8$ ). A significant increase in survival upon treatment with 264RAD is evident. Hazard ratio (HR) = 4.92 (CI: 1.04–23.28); log-rank  $p = 0.028$ . (C) PDAC tumours from KDC mice treated with IgG isotype control and gemcitabine or 264RAD and gemcitabine were analysed using immunohistochemistry for markers of tumourigenesis. Representative images of staining for Ki67, cleaved caspase-3 (C-Casp-3), cytokeratin 19 (CK19), alpha-smooth muscle actin ( $\alpha$ SMA), collagen, endomucin Endo), phosphorylated Smad3 (pSmad3), nuclear Smad4, and phosphorylated Erk (pErk) are displayed. Scale bar = 50  $\mu$ m. (D) Quantification of staining using image analysis as described in the Materials and methods section. Data show the mean  $\pm$  SEM of three tissue samples per group. Student's  $t$ -test, \* $p = 0.05$ .

*et al* that suggested a tumour-suppressive role of  $\alpha v \beta 6$  in PDAC [12]. Moreover, immunohistochemistry results revealed that tumours from KDC mice treated with 264RAD had significantly reduced proliferation (Ki67), tumour growth signalling (pErk), blood vessel density

(endomucin), and TGF $\beta$  signalling (nuclear Smad4), and showed a trend towards reduced desmoplasia (reduced  $\alpha$ SMA-expressing myofibroblasts and collagen deposition). Thus, antibody blockade of  $\alpha v \beta 6$  correlated with both direct anti-tumour cell effects (reduced

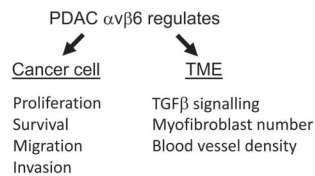


Figure 6. Mechanisms by which integrin  $\alpha\text{v}\beta 6$  promotes pancreatic ductal adenocarcinoma. Our data show that  $\alpha\text{v}\beta 6$  promotes PDAC by modulating the behaviour of both the cancer cells and the tumour microenvironment.

pErk, Ki67) and generation of a less tumour-permissive stroma. Together with reduced migratory and invasive capacity, these effects combine to suppress PDAC growth and increase overall survival. Figure 6 shows our current model of how  $\alpha\text{v}\beta 6$  affects PDAC growth.

Of all cancer conditions, PDAC remains the one that is most desperate for novel therapeutic strategies. As  $\alpha\text{v}\beta 6$  is expressed by the majority of human PDACs, and as our clinical, functional, and pre-clinical animal studies combine to show that this integrin actively promotes this disease, we must consider  $\alpha\text{v}\beta 6$  as a promising target for the therapy of PDAC in combination with conventional therapies.

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## Author contributions statement

JFM conceived and directed the study, and wrote the final manuscript. All the authors critically assessed, edited, and approved the final manuscript. JFM, HMK, OJS, and STB supervised and coordinated the study, and analysed the data. SV, CSR, CWS, AD, and KMM performed the biological experiments, collected and analysed data, and contributed to writing. TRJE, OJS, JPM, SK, and CSR generated, supervised, and analysed the data from the KDC transgenic studies. PB, DC, NBJ, AB, and CC generated databases used in the study. SH, AB, CC, and JPM performed statistical analyses on clinical and pre-clinical data. AS and RL collected and supplied clinical material and associated clinical data for

immunohistochemistry studies on Verona samples. SK generated the KDC mouse.

## References

1. Cancer Research UK (CRUK). Pancreatic Cancer Incidence Statistics. Cancer Research UK 2017. [Accessed 20 June 2019]. Available at: <http://www.cancerresearchuk.org/about-cancer/pancreatic-cancer/survival>
2. Conroy T, Gavaille C, Samalin E, *et al.* The role of the FOLFIRINOX regimen for advanced pancreatic cancer. *Curr Oncol Rep* 2013; **15**: 182–189.
3. Conroy T, Desseigne F, Ychou M, *et al.* FOLFIRINOX versus gemcitabine for metastatic pancreatic cancer. *N Engl J Med* 2011; **364**: 1817–1825.
4. Sipos B, Hahn D, Carceller A, *et al.* Immunohistochemical screening for  $\beta 6$ -integrin subunit expression in adenocarcinomas using a novel monoclonal antibody reveals strong up-regulation in pancreatic ductal adenocarcinomas *in vivo* and *in vitro*. *Histopathology* 2004; **45**: 226–236.
5. Hamidi H, Ivaska J. Every step of the way: integrins in cancer progression and metastasis. *Nat Rev Cancer* 2018; **18**: 533–548.
6. Hynes RO. Integrins: bidirectional, allosteric signaling machines. *Cell* 2002; **110**: 673–687.
7. Breuss JM, Gallo J, HM DL, *et al.* Expression of the beta 6 integrin subunit in development, neoplasia and tissue repair suggests a role in epithelial remodeling. *J Cell Sci* 1995; **108**: 2241–2251.
8. Moore KM, Thomas GJ, Duffy SW, *et al.* Therapeutic targeting of integrin  $\alpha\text{v}\beta 6$  in breast cancer. *J Natl Cancer Inst* 2014; **106**: 169.
9. Hazelbag S, Kenter GG, Gorter A, *et al.* Overexpression of the  $\alpha\text{v}\beta 6$  integrin in cervical squamous cell carcinoma is a prognostic factor for decreased survival. *J Pathol* 2007; **212**: 316–324.
10. Bates RC. The  $\alpha\text{v}\beta 6$  integrin as a novel molecular target for colorectal cancer. *Future Oncol* 2005; **1**: 821–828.
11. Elayadi AN, Samli KN, Prudkin L, *et al.* A peptide selected by biopanning identifies the integrin  $\alpha\text{v}\beta 6$  as a prognostic biomarker for nonsmall cell lung cancer. *Cancer Res* 2007; **67**: 5889–5895.
12. Hezel AF, Deshpande V, Zimmerman SM, *et al.* TGF- $\beta$  and  $\alpha\text{v}\beta 6$  integrin act in a common pathway to suppress pancreatic cancer progression. *Cancer Res* 2012; **72**: 4840–4845.
13. McShane LN, Altman DG, Sauerbrei W, *et al.* Reporting recommendations for tumour MARKER prognostic studies (REMARK). *Br J Cancer* 2005; **3**: 387–391.
14. Collisson EA, Sadanandam A, Olson P, *et al.* Subtypes of pancreatic ductal adenocarcinoma and their differing responses to therapy. *Nat Med* 2011; **17**: 500–503.
15. Stratford JK, Bentrem DJ, Anderson JM, *et al.* A six-gene signature predicts survival of patients with localized pancreatic ductal adenocarcinoma. *PLoS Med* 2010; **7**: e1000307.
16. Winter C, Kristiansen G, Kersting S, *et al.* Google goes cancer: improving outcome prediction for cancer patients by network-based ranking of marker genes. *PLoS Comput Biol* 2012; **8**: e1002511.
17. Zhang G, Schetter A, He P, *et al.* DPEP1 inhibits tumour cell invasiveness, enhances chemosensitivity and predicts clinical outcome in pancreatic ductal adenocarcinoma. *PLoS One* 2012; **7**: e31507.
18. Froeling FE, Mirza TA, Feakins RM, *et al.* Organotypic culture model of pancreatic cancer demonstrates that stromal cells modulate E-cadherin, beta-catenin, and Ezrin expression in tumour cells. *Am J Pathol* 2009; **175**: 636–648.
19. Eberlein C, Kendrew J, McDaid K, *et al.* A human monoclonal antibody 264RAD targeting  $\alpha\text{v}\beta 6$  integrin reduces tumour growth and metastasis, and modulates key biomarkers *in vivo*. *Oncogene* 2013; **32**: 4406–4416.

20. Thomas GJ, Lewis MP, Hart IR, *et al.*  $\alpha\beta6$  integrin promotes invasion of squamous carcinoma cells through up-regulation of matrix metalloproteinase-9. *Int J Cancer* 2001; **92**: 641–650.
21. Haider S, Wang J, Nagano A, *et al.* A multi-gene signature predicts outcome in patients with pancreatic ductal adenocarcinoma. *Genome Med* 2014; **6**: 105.
22. Crowder MJ, Hand DJ. *Analysis of Repeated Measures* (1st edn). Chapman and Hall: London, 1990.
23. Olive KP, Jacobetz MA, Davidson CJ, *et al.* Inhibition of Hedgehog signaling enhances delivery of chemotherapy in a mouse model of pancreatic cancer. *Science* 2009; **324**: 1457–1461.
24. Hwang RF, Moore T, Arumugam T, *et al.* Cancer-associated stromal fibroblasts promote pancreatic tumour progression. *Cancer Res* 2008; **68**: 918–926.
25. Hingorani SR, Wang L, Multani AS, *et al.* *Trp53<sup>R172H</sup>* and *Kras<sup>G12D</sup>* cooperate to promote chromosomal instability and widely metastatic pancreatic ductal adenocarcinoma in mice. *Cancer Cell* 2005; **7**: 469–483.
26. Inman GJ. Switching TGF $\beta$  from a tumour suppressor to a tumour promoter. *Curr Opin Genet Dev* 2011; **21**: 93–99.
27. Ahmed S, Bradshaw A-D, Geta S, *et al.* The TGF $\beta$ /Smad4 signalling pathway in pancreatic carcinogenesis and its clinical significance. *J Clin Med* 2017; **6**: 5–15.
28. David CJ, Huang Y-H, Chen M, *et al.* TGF $\beta$  tumour suppression through a lethal EMT. *Cell* 2016; **164**: 1015–1030.
29. Singh A, Greninger P, Rhodes D, *et al.* A gene expression signature associated with ‘K-Ras addiction’ reveals regulators of EMT and tumour cell survival. *Cancer Cell* 2009; **15**: 489–500.
30. Thomas GJ, Lewis MP, Whawell SA, *et al.* Expression of the  $\alpha\beta6$  integrin promotes migration and invasion in squamous carcinoma cells. *J Invest Dermatol* 2001; **117**: 67–73.
31. Kidger AM, Keyse SM. The regulation of oncogenic Ras/Erk signalling by dual-specificity mitogen activated protein kinase phosphatases (MKPs). *Semin Cell Dev* 2016; **50**: 125–132.

### SUPPLEMENTARY MATERIAL ONLINE

**Figure S1.** Generation and validation of a human PDAC tumour-mimetic using  $\alpha\beta6$ -positive PDAC cells combined with pancreatic stellate cells

**Figure S2.** Survival and individual tumour growth curves of antibody-treated CFPac1/PS1 subcutaneous xenograft mouse models of pancreatic cancer

**Figure S3.** Characterisation of pancreatic cancer progression in KDC mice

**Table S1.** Distribution of *ITGB6* log<sub>2</sub> mRNA abundance across the clinical cohorts

**Table S2.** Integrin screening of a panel of PDAC cell lines by flow cytometry